INCORPORATION OF N-ACETYL GLUCOSAMINE INTO LIPID LINKED OLIGOSACCHARIDES

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SUMMARY: When beef liver microsomes are labeled with UDP-[^{3}H]N-acetyl glucosamine, three different lipid-bound saccharides are labeled: dolichol pyrophosphoryl-GlcNAc, dolichol pyrophosphoryl-$(GlcNAc)_2$ and a previously uncharacterized compound (component III). Incubation with UDP leads to the disappearance of dolichol pyrophosphoryl-$(GlcNAc)_2$ from microsomes prelabeled with UDP-^{3}H-N-acetyl glucosamine. This result provides further support for the suggestion of Leloir et al. (6) that dolichol pyrophosphoryl-$(GlcNAc)_2$ is synthesized from dolichol pyrophosphoryl-GlcNAc and UDP-N-acetyl glucosamine. Component III cannot be discharged with UMP or UDP. It elutes from DEAE-cellulose exactly as the other components. The saccharide portion of component III has a molecular weight of 800-900 and contains glucose in addition to N-acetyl glucosamine.

Mammalian cells contain a membrane associated system for the biosynthesis of glycoprotein which has a requirement for dolichol, a polyisoprenoid lipid. Dolichol is a carrier for the assembly of oligosaccharide chains which are then transferred to a protein acceptor (cf. 1). The initial step in this pathway is the formation of Dol-P-P-GlcNAc from UDP-GlcNAc and Dol-P by the following reaction (2,3,4,5):

$$\text{Dol-P} + \text{UDP-GlcNAc} \rightleftarrows \text{Dol-P-P-GlcNAc} + \text{UMP} \qquad [1]$$

The next step in the pathway is presumed to be the formation of Dol-P-P-$(GlcNAc)_2$. Leloir et al. (6) have isolated this compound from microsomes previously labeled in vitro with UDP-[^{14}C]GlcNAc, and have shown that the specific activities of the reducing and non-reducing N-acetyl glucosamine residues of released disaccharide are different. On the basis of these data they ruled out the possibility that Dol-P-P-$(GlcNAc)_2$ is synthesized by the reac-

Abbreviations: dolichol phosphate, Dol-P; dolichol pyrophosphoryl N-acetyl glucosamine, Dol-P-P-GlcNAc; dolichol pyrophosphoryl di-N-acetylchitobiose, Dol-P-P-$(GlcNAc)_2$.

tion of two molecules of Dol-P-P-GlcNAc (although it is possible that the two sugars arose from separate pools of Dol-P-P-GlcNAc, e.g., protein bound versus membrane lipid phase). Instead they suggested the following reaction:

$$\text{Dol-P-P-GlcNAc} + \text{UDP-GlcNAc} \rightleftarrows \text{Dol-P-P-(GlcNAc)}_2 + \text{UDP} \qquad [2]$$

Several alternate pathways can be envisioned which are also consistent with these data. We considered it important, therefore, to obtain direct evidence for reaction [2]. In this paper we provide support for reaction [2] by demonstrating the UDP-dependent discharge of N-acetyl glucosamine from Dol-P-P-$(GlcNAc)_2$. In the course of this work we discovered that a third liposaccharide was labelled during incubation of microsomes with UDP-[^{14}C] GlcNAc. A preliminary characterization of this component is presented.

MATERIALS AND METHODS: UDP-[^{3}H]N-acetyl glucosamine and NaB^3H_4 were obtained from New England Nuclear. N',N'-diacetylchitobiose was prepared by partial hydrolysis of chitin followed by gel filtration chromatography (7). Beef liver microsomes were isolated using the Sharples technique (8). The incubation of microsomes with UDP-[^{3}H]GlcNAc and the subsequent fractionation of labeled products by means of a two phase $CHCl_3$:MeOH extraction and column chromatography on DEAE-cellulose and sephadex G-15 is described in detail elsewhere (9).

Intact saccharides were released from dolichol by drying the liposaccharide, then sonicating in 0.1 M HCl for 15 min, and finally heating at 80° for 20 min (9). Hydrolysis of the saccharides to individual monosaccharides was accomplished by treatment in 4 N HCl at 100°for 4 hours. Paper chromatography of sugars was conducted on Whatman #1 paper, previously treated with 0.2 M $Na_2B_4O_7$ in n-butanol:pyridine:H_2O, 6:4:3 (6).

Treatment of saccharides with tritiated $NaBH_4$ was conducted as described by Leloir et al. (6). Unreacted NaB^3H_4 and spurious label were removed by passing the solution through an activated charcoal column followed by passage through both a Dowex-1 and a Dowex-50 column (10). The identification of the resulting alditols was accomplished by chromatography on a Kieselguhr thin layer plate developed in n-butanol:acetone:phosphate buffer, pH 5 (0.1 M), 40:50:10 (11).

RESULTS AND DISCUSSION: The isolation and characterization of the different liposaccharides which are labeled with UDP-[^{3}H]GlcNAc was accomplished as follows. Beef liver microsomes were incubated with UDP-[^{3}H]GlcNAc for 15 min at 24°. The reaction was stopped by the addition of 2 volumes of $CHCl_3$MeOH:H_2O (60:40:8). Centrifugation separated this mixture into an organic phase, an aqueous phase, and an interphase pellicle. The interphase

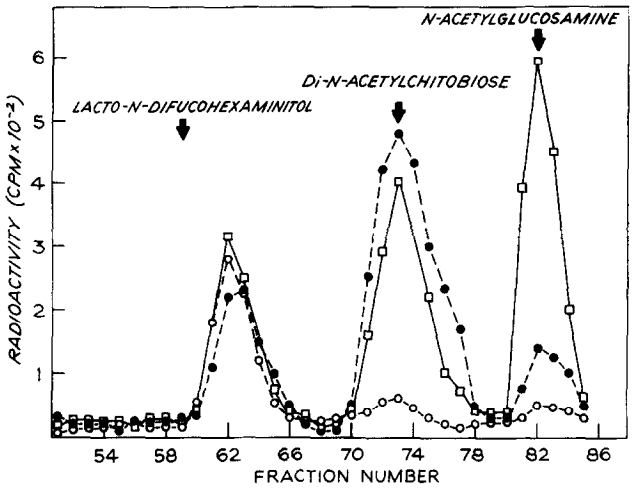

Figure 1. Chromatography on sephadex G-15 of the saccharide released from microsomal lipids which had been labeled with UDP-[^{3}H]GlcNAc. The dashed line indicates samples in which the labeling with UDP-[^{3}H]GlcNAc was followed by a 30 min incubation with either 1 mM UDP (O--O or 1 mM UMP (●--●). The solid line (●—●) indicates the control incubation in absence of added UMP or UDP.

pellicle was removed, and the liposaccharides extracted from it with $CHCl_3$: CH_3OH:H_2O (1:1:0.3). This extract was loaded onto a DEAE-cellulose column (7). The column was eluted first with $CHCl_3$:CH_3OH:H_2O (1:1:0.3), and the eluate discarded. A gradient of 0 to 0.1 N ammonium acetate in the same solvent was then initiated. The radioactivity eluted as a single peak. However, when a portion of this material was chromatographed on silica plates according to Ghalambor et al. (4) it was found to be heterogeneous.

To characterize the saccharide portion of the liposaccharides, we hydrolyzed the lipid-saccharide bond by mild acid hydrolysis, and removed the lipid with a $CHCl_3$ wash. The water soluble saccharides were separated on a sephadex G-15 column (Fig. 1, solid line). Three distinct radioactive peaks were obtained. Component I eluted as N-acetyl glucosamine, and co-migrated with it on paper chromatography (6). Component II eluted as di-N-acetyl-chitobiose, and chromatographed with this compound on paper (6). In addition to the two expected saccharides, a third radioactive component (component III) was found. Preliminary characterization of this compound is discussed elsewhere in this paper.

The data in Fig. 1 indicate that the saccharide product of the proposed reaction, the synthesis of Dol-P-P-$(GlcNAc)_2$ can be isolated and identified. It is possible therefore to examine the validity of reaction [2] by studying the reverse reaction. Thus, if reaction [2] reflects the manner in which Dol-P-P-$(GlcNAc)_2$ is synthesized, then UDP should discharge an N-acetyl glucosamine residue from Dol-P-P-$(GlcNAc)_2$. This possibility was tested. Microsomes were pre-labeled with UDP-[^{3}H]GlcNAc under standard conditions (9); 1 mM UDP was then added, and the incubation continued for 30 min. The liposaccharides were extracted and the saccharides released by mild acid hydrolysis, as above. The saccharides were chromatographed on sephadex G-15 (Fig. 1, 0---0). The data indicate that addition of UDP led to a discharge of label from di-N-acetylchitobiose. These data provide further support for the validity of reaction [2]. Figure 1 also shows that addition of UDP did not discharge label from component III. However, UDP did lead to a decrease of the amount of labeled N-acetyl glucosamine. This result is explained by the occurrence in beef microsomes of enzymes which rapidly convert UDP to UMP (12). UMP then reverses the reaction for the synthesis of Dol-P-P-GlcNAc, i.e., reaction [1]. This is clearly seen when UMP rather than UDP is added to microsomes prelabeled with UDP-[^{14}C]GlcNAc (Figure 1, ●---●). UMP releases label from Dol-P-P-GlcNAc but not from the other components. Palamarczyk and Hemming (5) have also reported on the UMP-dependent discharge of Dol-P-P-GlcNAc.

The structure and origin of the oligosaccharide we refer to as component III is unknown. The data in Fig. 1 suggest that it has a molecular weight of approximately 800-900 and that N-acetyl glucosamine residues are not removed by incubation of pre-labeled microsomes with UMP or UDP. To further characterize component III, it was hydrolyzed with strong acid, and then treated with NaB^3H_4. The resulting alditols were separated by Kieselguhr chromatography (10). As shown in Fig. 2, component III contains glucose as well as N-acetyl glucosamine. The oligosaccharide was treated first with

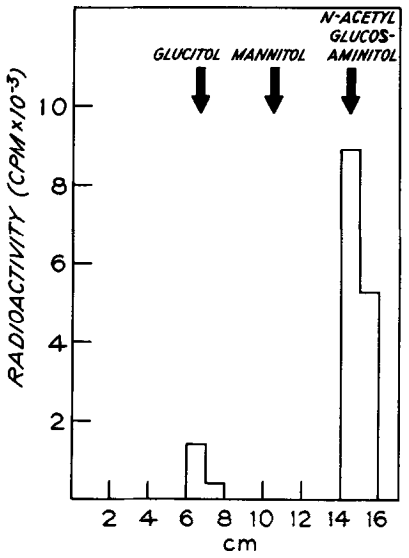

Figure 2. Thin-layer chromatography or Kieselguhr G of alditols derived from the component III oligosaccharide. [^{3}H]N-acetylglucosamine labeled component III (fractions 61-65 in Fig. 1) was collected and hydrolyzed by acid hydrolysis. The resulting monosaccharides were treated with NaB^3H_4. The [^{3}H]alditols were recovered as described in materials and methods and then separated on a Kieselguhr G thin layer plate run in n-butanol, acetone, 0.1 M phosphate (pH 5), 40:50:10.

α-glucosidase from yeast and then with β-glucosidase from almonds in an attempt to gain information on the location of glucose within it. Results of these experiments were inconclusive. Only approximately 30% of component III was degraded after a 10 hr incubation with each glucosidase and this could be the result of nonspecific hydrolysis. While the structure of component III is as yet uncertain, its existence suggests the presence in microsomes of an active glucose pool, probably in the form of Dol-P-glucose.

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REFERENCES

1. Lennarz, W. J. (1975) Science 188, 986-991.
2. Molnar, J., Chao, H. and Ikehara, Y. (1971) Biochim. Biophys. Acta 239, 401-410.
3. Behrens, N. H., Parodi, A. J., Leloir, L. F. and Krisman, C. R. (1971) Arch. Biochem. Biophys. 143, 375-383.

4. Ghalambor, M. A., Warren, C. D. and Jeanloz, R. W. (1974) Biochem. Biophys. Res. Comm. 56, 407-414.
5. Palamarczyk, G. and Hemming, F. (1975) Biochem. J. 148, 245-251.
6. Leloir, L. F., Staneloni, R. J., Carminatti, H. and Behrens, H. (1973) Biochem. Biophys. Res. Comm. 52, 1285-1292.
7. Arima, T. and Spiro, R. G. (1972) J. Biol. Chem. 247, 1836-1848.
8. Zakim, D. and Vessey, D. A. (1973) Methods Biochem. Anal. (D. Glick, ed.), Vol. 21, pp. 1-37, Wiley, New York.
9. Zatta, P., Zakim, D. and Vessey, D. A. (1976) Biochim. Biophys. Acta, in press.
10. McLean, C., Werner, D. A. and Aminoff, D. (1973) Anal. Biochem. 55, 72-84.
11. Lewis, B. A. and Smith, F. (1969) in Thin Layer Chromatography (E. Stahl, ed.) pp. 807-837, Springer-Verlag, New York.
12. Ernster, L. and Jones, L. C. (1962) J. Cell. Biol. 15, 563-578.